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RAPID COMMUNICATION

Saša Štefanić · Block S. Shaikenov · Peter Deplazes
Anke Dinkel · Paul R. Torgerson · Alexander Mathis

Polymerase chain reaction for detection of patent infections of *Echinococcus granulosus* ("sheep strain") in naturally infected dogs

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Abstract Polymerase chain reaction (PCR) for the identification of eggs of the tapeworm *Echinococcus granulosus* ("sheep strain") was evaluated with primers derived from mitochondrial sequences. Specificity of these primers was confirmed by investigating DNA of other strains of *E. granulosus* and of 14 helminth species which inhabit the intestines of dogs. This PCR assay was used to investigate 131 purged dogs from Kazakhstan. Eighteen dogs harboured *Echinococcus* worms, ten of them in mixed infections with *Taenia* spp. Coproantigen detection was positive in 15 and taeniid eggs could be recovered from 13 of these specimens. Eight of the egg-containing samples were positive in the PCR for *E. granulosus* and four in a *Echinococcus multilocularis* - specific PCR revealing one mixed infection. Egg-containing faeces from two dogs harbouring both *Taenia* spp. and *Echinococcus* spp. were negative in both PCRs. The combination of egg isolation and PCR will also be of value in epidemiological studies when investigating environmental samples.

Introduction

The larval stages of the tapeworm *Echinococcus granulosus* are the causative agent of hydatidosis (cystic echinococcosis; CE), one of the most important cestode infections causing morbidity and mortality in humans and significant economic losses in livestock. The parasite is perpetuated in life cycles with carnivores, primarily dogs, as definitive hosts, which harbour the adult egg-producing stages in the small intestine, and herbivorous and omnivorous species, in which the larval stages develop in the liver, lungs and other internal organs after ingestion of infective eggs. CE occurs on all continents with the highest prevalences being found in the Mediterranean basin, Eastern Europe, Central Asian Republics, China, North Africa and South America (Eckert et al. 2001a). Control programmes have been implemented with variable success in many regions with high incidence of CE (e.g. Cyprus, China, Chile, Spain), and the disease could be eradicated in some island countries (Iceland, New Zealand, Tasmania, Falkland Islands, part of Cyprus) (Torgerson and Budke 2003). However, there is strong evidence that this disease is an emerging problem in many countries worldwide (Eckert and Deplazes 2003). For example, the incidence of human CE has increased three- to fourfold in the last 10 years in Kazakhstan and Kyrgystan (Torgerson et al. 2002; 2003).

Phenotypic and genetic variation have been observed in isolates of *E. granulosus* from different species of intermediate hosts, and these host-adapted strains are designated according to their most commonly identified host (e.g. "sheep strain", "horse strain", "cattle strain", "camel strain", "pig strain"). Human CE is commonly caused by the "sheep strain" of *E. granulosus*, whereas other strains, such as the "horse strain" and the "pig strain", are considered to have low or no infectivity to man (Thompson and McManus 2002).

A prerequisite for initiating control programmes is an understanding of the transmission biology of *E. granulosus*, and the epidemiological characteristics in a given area include the assessment of baseline data of infections of definitive and intermediate hosts. Methods for the detection of tapeworm infections in definitive hosts have been reviewed and discussed comprehensively in recent publications (Eckert et al. 2001b; Fraser et al. 2002). Although post mortem diagnosis (sedimentation and counting technique) remains the "gold standard" due to

S. Štefanić · P. Deplazes · P. R. Torgerson · A. Mathis (✉)
Institute of Parasitology, University of Zürich,
Winterthurerstrasse 266a, 8057 Zürich,
Switzerland
E-mail: alexander.mathis@access.unizh.ch
Tel.: +41-1-6358536
Fax: +41-1-6358907

B. S. Shaikenov
Institute of Zoology, Kazakh Academy of Sciences,
Academogorodok, Almaty, Kazakhstan

A. Dinkel
Department of Parasitology, University of Hohenheim,
70599 Stuttgart, Germany

its high sensitivity (close to 100%) and specificity (99%) (Eckert et al. 2001b), *intra vitam* diagnosis is preferred for canine *E. granulosus* infections. In the past, purgation of dogs using arecoline hydrobromide was the major diagnostic approach. However, this procedure is laborious, costly, biohazardous and suffers from low sensitivity (65%) after a single dose application (Schantz et al. 1995). As an alternative, coproantigen detection by enzyme-linked immunosorbent assay (CA-ELISA) has been developed and evaluated by several working groups for the detection of *E. granulosus* intestinal infections (Allan et al. 1992; Deplazes et al. 1992; Malgor et al. 1997). CA-ELISAs, which are fast tests, are valuable diagnostic tools for large epidemiological screenings, and they can also be applied to field faecal samples because of the stability of coproantigens (Deplazes et al. 2003). The sensitivities of such tests, which are dependent on parasite burdens, are high in animals harbouring many parasites. However, due to false-positive reactions and cross-reactivity with *Taenia* spp., the specificities of these tests are limited (80%–96%; Allan et al. 1992; Deplazes et al. 1992; Malgor et al. 1997; Christofi et al. 2002). Therefore, positive predictive values are relatively low, particularly in situations of low endemicity (Christofi et al. 2002), and positive ELISA results need further confirmation. Microscopical detection of *E. granulosus* eggs is not suitable for this purpose as they are morphologically indistinguishable from those of other taeniids. Egg identification by *E. granulosus* -specific monoclonal antibodies has been described (Craig et al. 1986) but this method has not been used in further epidemiological studies. Molecular biological methods are feasible for this purpose, but no such test has been described for diagnosis of *E. granulosus* eggs recovered from faecal or from environmental samples as yet. Here, we report on the diagnosis of *E. granulosus* based on purged material from naturally infected dogs and on our evaluation of a polymerase chain reaction (PCR) assay for the identification of eggs of *E. granulosus* ''sheep strain''.

Materials and methods

Sample collection

Between September and November 2002, 131 rural dogs in Kazakhstan were purged by administering up to 10 mg/kg of arecoline hydrobromide in an aqueous solution. For safety reasons, faeces were stored at −80°C for at least 5 days before being further processed. In 18 of the purged dogs, *Echinococcus* spp. worms were detected, rinsed in water and stored in 70% ethanol at −20°C until microscopical and molecular examination. Eggs from mature worms were obtained after mechanical disruption of worms that were kept at −80°C for at least 5 days.

Coproantigen-ELISA

A commercially available coproantigen ELISA (Chekit Echinotest; Bommeli, Liebefeld-Bern, Switzerland), which is designed for the detection of *E. granulosus* and *Echinococcus multilocularis* coproantigens in dogs, foxes and cats, was performed according to the manufacturer's instructions, with the exception that 2 g of material was diluted 1:4 in the kit's sample dilution buffer and that the samples were not run in duplicates. After centrifugation of the sample suspensions (3,000 *g* at room temperature for 10 min), the supernatants were used for ELISA while the sediments were stored at 4°C for taeniid egg isolation.

Isolation of taeniid eggs; DNA isolation

The procedure for isolation and microscopical detection of taeniid eggs was carried out with all faecal specimens by a combination of sequential sieving and flotation of the eggs in zinc chloride solution and egg detection using an inverted microscope as previously described (Mathis et al. 1996). DNA isolation from samples containing taeniid eggs was performed with all the sediment if < 30 eggs were present or with aliquots containing approximately this number of eggs.

Initially, we sought to improve the original method for obtaining DNA from eggs recovered from faeces (Mathis et al. 1996) which includes the use of organosolvents (phenol-chloroform extraction). To this end, eggs from 20 canine faecal samples, originating from the diagnostic unit of our institute and known to contain taeniid eggs, were isolated as described above. DNA from aliquots containing approximately 30 eggs was isolated either according to the published method (Mathis et al. 1996) or as follows: after alkaline lysis of the eggs and neutralisation as in the original method (Mathis et al. 1996), 20 µl proteinase K and 200 µl of lysis buffer of a commercial kit (Qiamp DNA mini kit; Qiagen, Hilden, Germany) were added to the 200-µl samples, and digestion was performed for 10 min at 56°C. Samples were then centrifuged (1 min, 13,000 *g*), and supernatants were transferred into new tubes. Into each tube, 50 µl Chelex beads (50% w/v in distilled water; Bio-Rad Laboratories, Hercules, Calif.) were added, and the tubes were kept rotating for 30 min at room temperature. After centrifugation as above, supernatants were transferred into new tubes to which 250 µl ethanol (100%) was added. After vortexing for 15 s, the samples were loaded onto the kit's columns and, after washing steps according to the kit's protocol, DNA was eluted in 100 µl of 10 mM TRIS-HCl, pH 8.3 and stored at −20°C until use.

Isolation of DNA from metacestodes and from worms was done with the above-mentioned Qiamp DNA mini kit according to the manufacturer's instructions.

PCR and sequencing

E. granulosus PCR

The primer pair for amplification of DNA of *E. granulosus* ''sheep strain'' was chosen from the sequence of the mitochondrial 12S rRNA gene (GenBank accession no. AF297617; primer sequences Eg1f, 5′-CAT TAA TGT ATT TTG TAA AGT TG-3′; Eg1r, 5′-CAC ATC ATC TTA CAA TAA CAC C-3′) yielding an amplicon of 255 bp. Amplification reactions were prepared in total volumes of 100 µl consisting of PCR buffer (50 mM KCl, 20 mM TRIS-HCl pH 8.4, 2.5 mM $MgCl_2$, 0.5% Tween 20), 0.2 mM of each dNTP (using dUTP instead of dTTP), 1 µM of each primer, 0.5 U uracil DNA glycosylase (UDG; Gibco BRL/Life Technologies, Gaithersburg, Md.). After 10-min incubations steps at 37°C and 94°C (to inactivate the UDG), respectively, 2.5 U Taq polymerase (Sigma-Aldrich, Buchs, Switzerland) were added using a ''hotstart''. Forty cycles of 30 s at 94°C, 30 s at 53°C and 45 s at 72°C were performed in a thermal cycler (DNA engine; MJ Research, Waltham, Mass.) with a final extension at 72°C for 10 min. Each sample was tested in triplicate, one with 25 µl sample solution, the second with 2 µl and the third using 2 µl spiked with 1 µl (10^5 copies) of a cloned, size-modified control target that was created using composite

primers (Celi et al. 1993). This control target, which detects amplification inhibition, yields an amplicon of 292 bp upon amplification, which is easily discriminated from the *E. granulosus* -specific product after gel electrophoresis. In each run, one negative control without DNA and one positive control (1 µl control plasmid DNA) were included. Amplicons were detected after electrophoresis of 15 µl of PCR mixtures in 1.5% agarose gel and staining with ethidium bromide (0.8 µg/ml). The specimens which lacked the internal control band of 292 bp were considered as inconclusive and were re-tested after a second purification using Qiagen columns.

DNA sequencing was performed directly on amplicons by automated means by a private company (Microsynth, Balgach, Switzerland).

E. multilocularis PCR

E. multilocularis -specific DNA was detected by using a modified PCR (Dinkel et al. 1998; Stieger et al. 2002) with the primer pair EM-H15:5′-CCA TAT TAC AAC AAT ATT CCT ATC-3′ and EM-H17:5′-GTG AGT GAT TCT TGT TAG GGG AAG-3′, which amplifies a product of 200 bp from the *E. multilocularis* 12S rRNA gene. Amplification reactions were prepared in the same manner as for *E. granulosus* PCR (see above) including a corresponding internal control target (A. Mathis, unpublished data).

Specificity of the *E. granulosus* PCR

In order to test the specificity of the *E. granulosus* primers, DNA from a wide variety of helminths was subjected to PCR: *E. granulosus* strains (as determined by mitochondrial genomic markers, Bowles et al. 1995): ''sheep strain'', ''horse strain'', ''cattle strain'', ''camel strain'', ''pig strain''; *E. multilocularis* (five isolates), *Echinococcus vogeli* (1), *Taenia taeniaeformis* (3), *Taenia pisiformis* (2), *Taenia crassiceps* (2), *Taenia multiceps* (3), *Taenia hydatigena* (1), *Taenia saginata* (2), *Taenia solium* (1), *Dyphillobothrium latum* (1), *Dypillidium caninum* (2), *Trichuris vulpis* (3), *Toxocara canis* (3), *Uncinaria stenocephala* (1). The presence and integrity of DNA in all these samples was confirmed by PCR with a set of universal eukaryotic primers (forward, 5′-CTA GGA TTA GAT ACC CTA T-3′ and reverse, 5′-AAG AGC GAC GGG CGA TGT GT-3′) (O'Neill et al. 1992) after amplification (1 min at 94°C, 1 min at 52°C, 1 min at 72°C) for 35 cycles.

Results and discussion

E. granulosus PCR

The specificity of the primers Eg1f /Eg1r for *E. granulosus* ''sheep strain'' was 100% as shown by examining DNA from five other strains of *E. granulosus* and from 14 different helminth species, including five samples from *E. multilocularis* and one from *E. vogeli*, which all remained PCR-negative.

Diagnosis on purged dog material

Purged samples from 131 dogs from a known *E. granulosus* -endemic area in south-east Kazakhstan were chosen for test validation. *Echinococcus* spp. worms were detected in 18 samples, in eight cases as single infections and in ten cases as mixed infections with *Taenia* spp. (Table 1). Taeniid eggs could be recovered from five of these eight single infections, and PCR revealed the presence of *E. granulosus* in four out of these five cases. Sequencing in one instance confirmed that the 255 bp amplicon corresponded to the *E. granulosus* ''sheep strain'' mitochondrial 12S rRNA gene. The fifth dog, which harboured gravid *Echinococcus* worms, was PCR-positive for *E. multilocularis*, which is the first demonstration of *E. multilocularis* in dogs of this area.

Taeniid eggs were obtained from eight of the ten mixed infections (*Taenia* spp. and *Echinococcus* spp.). PCR was positive in four cases for *E. granulosus*, in three cases for *E. multilocularis* with one dog being positive for both these tapeworms. Egg-containing faeces from two dogs were negative in both PCRs.

Single infections with *Taenia* spp. were determined in 14 cases upon examination of purged material. PCR for *E. granulosus* was negative in all of these samples which contained eggs (9), but the presence of *E. multilocularis* was proven by PCR in one dog. The relative

Table 1 Diagnosis of *Echinococcus* spp. in naturally infected, rural dogs by purgation, coproantigen enzyme-linked immunosorbent assay (*CA-ELISA*) and polymerase chain reaction (*PCR*) on taeniid eggs recovered from purged material

Purgation results		CA-ELISA[a] (Positive/no. tested)	Taeniid eggs[b] (Positive/no. tested)	PCR[c] (Positive/no. tested)	
				E. granulosus	*E. multilocularis*[d]
Echinococcus spp.	(n = 8)	7/8	5/8	4/5	1/5
Mixed infections[e]	(n = 10)	8/10	8/10	4/8	3/8
Taenia spp.	(n = 14)	5/14	9/14	0/9	1/9
No taeniids	(n = 99)	17/99	9/99	0/9	1/9
Total	(n = 131)	37/131	31/131	8/31	6/31

[a]Chekit Echinotest (Bommeli, Liebefeld-Bern, Switzerland)
[b]As described by Mathis et al. (1996)
[c]PCR was performed only on samples with microscopically detected taeniid eggs
[d]Modified PCR after Dinkel et al. (1998)
[e]*Echinococcus* spp. and *Taenia* spp

low sensitivity of purgation was suggested by further analysis of the 99 purgation-negative (no taeniids) results. Taeniid eggs were recovered in nine of these samples, and in one case an infection with *E. multilocularis* was substantiated by PCR.

CA-ELISA was positive with all samples that were positive by PCR for either *E. granulosus* ''sheep strain'' or *E. multilocularis*. Hence, the diagnostic strategy we use for *E. multilocularis* in foxes, namely CA-ELISA followed by confirmation of ELISA-positive results by egg isolation and PCR on recovered eggs (Mathis and Deplazes 2002), seems to be an efficient one also in the case of canine *E. granulosus*.

Twelve of the total 14 *Echinococcus*-positive PCR results obtained with taeniid eggs, including a mixed infection with *E. granulosus* and *E. multilocularis*, could be confirmed by microscopical investigations on worms recovered from the purged material. The two infections with *E. multilocularis* in animals which were purgation-negative for *Echinococcus* worms were confirmed by sequencing of the amplicons. Hence, no false-positive results were obtained with PCRs specific for *E. granulosus* and *E. multilocularis*, respectively.

Two recent publications report on the use of PCR for identification of *E. granulosus* infections in final hosts, neither assay allowing identification of the strain. Cabrera et al. (2002) derived degenerate primers from aligned mitochondrial sequences yielding a theoretical specificity for the six strains of *E. granulosus* considered in primer design and for *E. oligarthrus* and *E. vogeli*. Upon evaluation with eggs obtained directly from gravid proglottids of a few helminths, amplification products were obtained with one isolate of *E. granulosus*, but not with single isolates of *E. multilocularis*, *T. hydatigena*, *T. saginata* , *Hymenolepis nana* and *Dipylidium caninum*. The test has not been further validated for diagnostic use on faecal or environmental material. Abbasi et al. (2003) used the information on a newly identified repeated sequence from *E. granulosus* ''sheep strain'' to design PCR primers. The evaluation of their PCR, which yields a banding pattern upon analysis of amplicons on agarose gels in positive cases, revealed 100% sensitivity and specificity with DNA samples extracted from 0.3 ml of faeces from 34 infected and 18 non-infected dogs. The primers also amplified DNA from metacestode material of other *E. granulosus* strains, albeit at a lower sensitivity and showing different banding patterns.

DNA isolation

A new protocol was developed for DNA extraction from taeniid eggs isolated from faecal samples. This protocol, which includes the use of Chelex-100 resin in combination with a commercial DNA isolation kit, has successfully been applied in our laboratory with fox faecal samples (unpublished data). When evaluated with dog faecal samples, this modified method proved its superiority: as shown in Fig. 1, PCR with DNA obtained with the new method was more efficient than with DNA isolated with the old standard method. Nine of the 20 samples with taeniid eggs were positive for *E. granulosus* by PCR with DNA from both methods when employing 2 µl of DNA sample. However, with DNA isolated with the new method, signals were stronger and amplicons were also obtained when using 25 µl in the amplification reaction, in contrast to reactions with the same volume of DNA obtained with the old method which yielded PCR-negative results, indicating the presence of PCR-inhibitory substances.

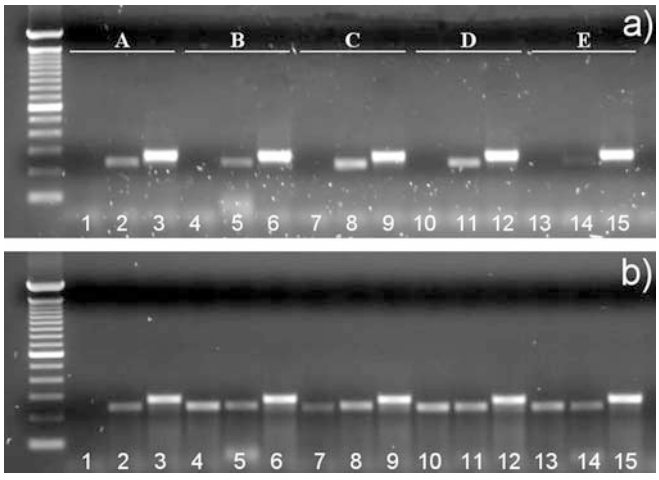

Fig. 1a, b *Echinococcus granulosus* polymerase chain reaction on taeniid eggs recovered from five (*A–E*) canine faecal samples. DNA isolation: **a** standard method including organosolvent extraction; **b** modified method using Chelex/commercial DNA isolation kit. *Lanes 1, 4, 7, 10, 13* High (25 µl) sample volume; *lanes 2, 5, 8, 11, 14* low (2 µl) sample volume; *lanes 3, 6, 9, 12, 15* low (2 µl) sample volume with internal control target

The inclusion of an internal control in the amplification reactions allows one to check for PCR-inhibitory effects in the DNA solutions and hence to clearly distinguish between false and true negative results. From the 31 faecal samples of dogs that were positive for taeniid eggs in our study and which were processed for PCR according to the novel DNA isolation protocol, inhibition was obvious in as many as eight samples (25.8%). All these samples consisted of a large pellet after sieving, and five of these samples contained less than eight taeniid eggs. According to our strategy, all the sediment from the original 2 g of these faecal materials was employed for DNA isolation. However, after a second round of DNA purification with the commercial kit, PCR inhibition could no longer be detected, and PCR was positive with a specimen which contained only two eggs. Hence, in contrast to our experience with DNA isolation from fox faeces with this method, which did not result in inhibited PCRs, DNA isolation from canine faeces required some improvement in order to eliminate the, as yet unknown, factors which impair DNA amplification. The fact that a second round of DNA purification resulted in clear-cut PCR results

indicates that the default washing steps recommended in the manufacturer's instructions are not sufficient with DNA from dog faeces.

In conclusion, we have evaluated a PCR for identifying *E. granulosus* "sheep strain" from taeniid eggs, showing its high specificity with no false-positive results with DNA from a variety of other helminths and with purged material containing taeniid eggs. The method of concentrating taeniid eggs with subsequent molecular identification by use of the PCR described here specific for *E. granulosus* "sheep strain" will also be of value in epidemiological studies when investigating environmental samples to which no other diagnostic technique can be applied.

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